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(71) Applicants *(for all designated States except US)*: SANDOZ LTD. [CH/CH]; Lichtstrasse 35, CH-4002 Basle (CH). NEW ENGLAND DEACONESS HOSPITAL CORPORATION [US/US]; 185 Pilgrim Road, Boston, MA 02215 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BACH, Fritz, H. [AT/US]; 8 Blossom Lane, Manchester-By-The-Sea, Boston, MA 01966 (US). WRIGHTON, Christopher [GB/US]; Unit 5, 17 Stearns Road, Brookline, MA 02146 (US).

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(57) Abstract

A method to genetically modify mammalian cells to render them less susceptible to an inflammatory or immunological stimulus, particularly to alleviate thrombogenicity in endothelial cells subject to inflammatory or immune activation, is described, comprising genetically modifying the cells by inserting therein DNA encoding thrombomodulin and thereby expressing functional thrombomodulin from these cells under cellular activating conditions. The method, which can be carried out either *in vivo* or *in vitro* (*ex vivo*), is indicated for use in transplantation as well as to treat systemic or local inflammatory conditions characterized by thrombosis.

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GENE THERAPY FOR TRANSPLANTATION AND INFLAMMATORY OR THROMBOTIC CONDITIONS

Field of the invention

The invention provides improvements in the field of gene therapy for transplantation and inflammatory and thrombotic conditions. In particular, it is concerned with genetic modification of endothelial cells to render them less susceptible to an inflammatory or immunological stimulus. It is also concerned with transplantation of genetically modified tissues or organs and most particularly is directed to a method of transplanting genetically modified endothelial cells or tissue or organs into a mammalian recipient of the same or different species, recombinant vectors for accomplishing same, and the cells, tissue or organs, as well as non-human transgenic animals, so modified.

Background of the invention

The endothelium consists of the layer of cells which form the lining of blood vessels, e.g. arteries, veins and capillaries. While the endothelium (also referred to as "vascular endothelium") was once thought to serve merely as a passive conduit for the blood and blood serum, it is now recognized that the condition of the endothelial tissue is critical for maintaining the normal anti-coagulatory state of circulating blood. In particular, the thrombomodulin (TM) protein which is manufactured by endothelial cells and is distributed as a surface glycoprotein throughout the endothelium of arteries, veins and capillaries, constitutes a multifunctional anti-coagulant molecule which is pivotal in the well-characterized Protein C coagulation pathway. In this pathway, thrombomodulin functions as a binding receptor of circulating thrombin, removing thrombin from circulation and thereby precluding it from, among other functions, clotting fibrinogen or activating platelets. Most importantly, once bound by thrombomodulin, the thrombin binds up circulating, inactive Protein C, which is thus converted to the activated form. The action of activated Protein C on still other co-factors of the coagulant pathway, i.e Protein S, ultimately assists in maintaining an anti-coagulatory steady-state.

In response to injury, inflammation or immunologic challenge, such as occurring in connection with allogeneic or xenogeneic transplantation of tissues or organs, as well as other inflammatory conditions such as vari us autoimmune diseases or sepsis, the endothelium is changed to a dysfunctional or "activated" pro-coagulatory state. An aspect of this conversion occurring in the presence of cytokines such as tumor necrosis factor (TNF or TNFα), is the loss or consumption of thrombomodulin from the endothelial cell surface. This reduction in thrombomodulin has been ascribed to downregulation at the transcriptional level [Yu et al., J. Biol. Chem. 2667 (1992) 23237-23247].

Thus, inflammatory or immunologic challenge, such as accompanies transplantation of tissues or organs, presents a significant threat of microvascular thrombosis, i.e. pathological clotting of the blood in the microvasculature of the endothelium, where thrombomodulin is ordinarily most active, and inflammation.

For the above reasons, in connection with implantable synthetic materials such as prosthetic arterial grafts, Ito and co-worker, for example, have used thrombomodulin protein as an adherent surface coating (USP 5'126'140). However, this approach has limited usefulness where live grafts are concerned.

The problem of endothelial cell dysfunction or "activation" in the organ transplant setting has been addressed largely by efforts to disable the host immunological response, such as with cytotoxic drugs, anti-metabolites, corticosteroids, non-specific immunosuppressants such as cyclosporine A, and the like.

There exists a critical need for a method to alleviate thrombogenicity accompanying activation of endothelial cells, and in particular, to prolong organ transplant survival, while minimizing toxicity and other adverse effects associated with large doses of immunosuppressants.

Summary of the invention

The invention utilizes gene therapy techniques to alleviate the thrombogenicity (i.e. the tendency to promote blood clotting) of vascular endothelial cells susceptible to an inflammatory or immunological stimulus.

Endothelial cells have been modified by rendering them capable of expressing functional thrombomodulin on a regulable or constitutive basis, whereby thrombomodulin expression is maintained even in the presence of endothelial cell activating factors. By "functional" is meant that the expressed thrombomodulin protein of said endothelial cell can bind thrombin in the cellular environment (for example, in the presence of blood of a graft recipient), and that the thrombomodulin-thrombin complex thus formed can catalyze the activation of zymogen (i.e. inactive) protein C to provide activated protein C. By "regulable" is meant that protein expression, whether increased or decreased, is dependent on the presence of, or addition of, a given substance. The term "regulable expression" includes "inducible expression", whereby gene expression is increased by addition of a stimulus. By "constitutive" is meant that protein expression is essentially independent of endothelial cell activation. Thus, constitutive expression of thrombomodulin by an endothelial cell is substantially free of down-regulation by TNFα or other activating factors. Likewise, a constitutive promoter of thrombomodulin is capable of directing transcription of the gene even in the presence of serum down-regulatory factors such as TNF.

Accordingly, the invention concerns a method of genetically modifying mammalian, preferably endothelial cells to render them less susceptible to an inflammatory or immunological stimulus, preferably by inserting into these cells, or the progenitors of these cells, DNA encoding functional thrombomodulin protein in operative association with a suitable promoter, whereby thrombomodulin is expressed under endothelial cellular activating conditions.

The invention also comprises a method of inhibiting thrombosis in a mammalian subject susceptible to an inflammatory or immune stimulus which comprises inserting into cells, tissue or an organ of the subject, DNA encoding thrombomodulin in operative association with a promoter, whereby functional thrombomodulin is expressed from these modified cells in the presence of cellular activating factors.

Preferably the cells or tissue are modified in vivo, i.e. while remaining in the body of the patient. Alternatively, cells or tissue may be extracted from the subject and transferred to in vitro culture, where they are genetically modified by insertion of DNA as described herein, and then grafted into the subject or a different recipient. The subject is a vertebrate, in particular a mammal, such as a pig, but may also be a primate, and in particular, a human. Such a therapy will be useful to relieve inflammatory conditions or immune conditions, such as autoimmune diseases, in a patient, especially where the patient is a human.

In a further aspect the invention comprises a method of transplanting donor allogeneic or xenogeneic endothelial cells, or tissue or organs containing endothelial cells to a mammalian recipient in whom such cells, tissue or organ are subject to inflammatory or immune activation, which comprises:

(a) genetically modifying these donor cells or progenitor cells thereof or tissue or organ by inserting therein DNA encoding thrombomodulin under the control of a promoter;
(b) implanting the resultant modified donor cells, tissue or organ into the recipient; and
(c) allowing expression from the resultant modified cells, tissue or organ of functionally active thrombomodulin.

Steps (a) and (b) may be carried out in either order; that is, the donor allogeneic or xenogeneic cells, tissue or organ may be modified or genetically engineered (e.g., by transfection, transduction, transformation or the like) prior to, or alternatively after, implantation into the recipient.

The promoter may be either constitutive or regulable, e.g. inducible.

For example, endothelial cells or tissue recruited from a pig may be genetically modified in vivo by insertion of DNA encoding human thrombomodulin under the control of a suitable promoter, and the modified cells or tissue, having been rendered "transgenic" thereby if modification has occurred in the germ line or alternatively, somatic recombinants if modification has occurred in the somatic cells, are then grafted into the recipient, e.g. a human. Once transplanted, the modified cells or tissue will express functional human thrombomodulin at the graft site, even in the presence of otherwise downregulatory factors such as TNF. Genetic modification of the donor mammal can be carried out at an embryonic stage, by well-known techniques, so as to produce a transgenic or somatic recombinant animal expressing the desired recipient protein. The donor, e.g. pig endothelial

cells or tissue can also be genetically modified by ex vivo means, whereby cells, tissues or organs extracted from the donor and maintained in culture are genetically modified as described above, and then transplanted to the recipient, where the graft can then express the desired functional recipient thrombomodulin substantially free of down-regulation in the presence of endothelial cell activating factors.

It is preferable that the genetic modification be done in vivo rather than in vitro (i.e. ex vivo). The preferred in vivo method is by raising up of a transgenic mammal, e.g. a pig, to be used as a source of donor cells, tissues and organs expressing the desired functional protein.

Particularly suitable promoters and vector constructs have been identified with which to modify endothelial cells or tissues, and in particular porcine endothelial cells [e.g. porcine aortic endothelial cells (PAEC) or porcine microvascular endothelial cells in general], so as to render them constitutive or regulable expressers of functional thrombomodulin.

In this regard, in one embodiment, a construct is described hereafter which comprises the thrombomodulin DNA in operative association with the Herpes simplex thymidine kinase (tk) promoter.

A retroviral construct is further utilized which comprises:

(a) a 5'-long terminal repeat (LTR) of a retrovirus such as Moloney murine leukemia virus (Mo-Mu-LV));

(b) a retroviral packaging signal such as Ψ, downstream from the LTR;

(c) DNA encoding thrombomodulin in operative association with the Herpes simplex thymidine kinase promoter downstream from the LTR; and

(d) a 3'-long terminal repeat.

The vector may also comprise at least a portion of the retroviral gag coding region (e.g. about 400 nucleotides thereof), generally downstream from the packaging signal sequence, i.e. normally ψ. The 3'-LTR also preferably includes a polyadenylation sequence. Preferably, a selectable marker such as the neo gene, conferring resistance to neomycin or an analog thereof, may be placed under the control of the 5'-LTR, generally downstream of the packaging signal sequence. An example of such a plasmid is pNTK-2.TM.CW1, illustrated in **FIG. 3.**

Adenoviral constructs may also be utilized, and are prepared e.g. as described hereunder. In particular, perfusion of diseased vessels with an adenoviral construct encoding TM can be ffected to introduce the TM into the endothelium in the diseased vessels.

According to a further aspect of the invention, there is provided graftable mammalian endothelial cells, tissue or organs comprising DNA encoding thrombomodulin under the control of a promoter, especially graftable endothelial cells, tissue or organs of a donor mammalian species, the cells, tissue or organs being modified to express thrombomodulin of a graft recipient species which is the same or a different species as the donor. Expression may be constitutive or regulable.

A further aspect of the invention provides for a non-human transgenic mammal comprising DNA encoding thrombomodulin of a different species, particularly having transplantable endothelial cells or tissue modified as described above, and a method of preparing a non-human transgenic mammal comprising DNA encoding thrombomodulin of a different species. An example of such a mammal is a transgenic mouse expressing human TM from its endothelial (or other) cells. A further example is a transgenic pig expressing human TM from its endothelial (or other) cells.

In an illustrative embodiment, the invention comprises a method of transplanting into a human, an organ comprising porcine endothelial cells genetically modified to express functional human thrombomodulin.

Description of the drawings

FIG. 1 Restriction map of plasmid pNTK-2.

FIG. 2a Schematic view of plasmid pUC19.TM15 encoding human TM.

FIG. 2b Schematic view of the insert of plasmid pUC19.TM.CW1
(in FIGS. 2a and 2b, the bold lines indicate plasmid backbone sequences).

FIG. 3 Schematic view of the expression vector pNTK-2.TM.CW1

FIG. 4a FACscan analysis of the NTK.TM.CW1 producer cell pool.

FIG. 4b Bar graph depicting relative expression levels of TM clones (arbitrary units) from limiting dilution of the NTK-2.TM.CW1 producer pool.

FIG. 5 Northern analysis of NTK.TM.CW1 transduced cells.

FIG. 6 Nuclease S1 analysis of thrombomodulin expression.

FIG. 7 Illustrated principle of the chromogenic assay for activated Protein C generation.

FIG. 8 Rate of S-2366 cleavage at 37°C:

dotted squares:	untransduced PAEC
lozenges:	NTK-2.TM.CW1 transduced and selected PAEC
dark squares:	EAhy926 cell line.

FIG. 9 Bar graph illustrating activity of thrombomodulin, as reflected by maximal rate of activated Protein C (APC) production, following treatment of cells with TNF.

FIG. 10 TM.CW1 comprising the native human thrombomodulin cDNA sequence (SEQ ID No.1) and NTK-2.TM.CW1 restriction analysis.

Definitions

"Graft", "transplant" or "implant" are used interchangeably to refer to biological material derived from a donor for transplantation into a recipient, and to the act of placing such biological material in the recipient.

"Host" or "recipient" refers to the body of the patient in whom donor biological material is grafted.

"Transgenic" refers to animals bearing foreign genes, independently of whether the gene is in the germ line or in the somatic cells.

"Allogeneic" (and "allograft") refer to the donor and recipient being of the same species. As a subset thereof, "syngeneic" refers to the condition wherein donor and recipient are genetically identical. "Autologous" refers to donor and recipient being the same individual. "Xenogeneic" (and "xenograft") refer to the condition where the graft donor and recipient are of different species.

"Mo-Mu-LTR": Moloney Murine Leukemia Virus long terminal repeat DNA sequence.

"polyA": DNA sequence encoding a polyadenylation signal.

"neo": the gene encoding neomycin phosphotransferase, which confers resistance to neomycin or neomycin analogs such as G418, which are toxic to most eukaryotic and other host cells.

"promoter" a DNA sequence that directs transcription of DNA into RNA.

"tk" Herpes Simplex thymidine kinase.

"PAEC" porcine aortic endothelial cells.

"HUVEC" human umbilical vein endothelial cells.

"EAhy926 HUVEC": immortalized human umbilical endothelial cell line expressing human TM constitutively.

"TNF": tumor necrosis factor.

"Thrombomodulin" (TM) refers to the natural thrombomodulin gene (including the cDNA thereof) or protein, and include derivatives thereof having variations in DNA (or amino acid) sequence (such as silent mutations or deletions) which do not prejudice the functionality of natural thrombomodulin (USP 5'273'962 and USP 5'256'770).

Detailed description of the invention

The gene encoding native thrombomodulin has been isolated and sequenced from several species, both in its generic form and as cDNA. The complete cDNA sequence for human thrombomodulin, derived from human umbilical vein endothelial cells, comprising an open reading frame of 1'725 base pairs, encoding a 60.3 kDa (M_r = 60'328) protein of 575 amino acids, is disclosed by Majerus, USP 4'912'207, and is also depicted for nucleotide positions 170 to 1'894 in **FIG. 10 (SEQ ID No.1)** hereof.

The invention broadly comprises rendering endothelial cells or tissue or organs less susceptible to activation or dysfunction in response to an immune challenge, by modification of these endothelial cells, tissue or organs by insertion therein of DNA encoding thrombomodulin in operative association with an appropriate promoter and expressing functional thrombomodulin from these modified cells at effective levels.

The promoter may be constitutive or regulable, e.g. operate in appropriately inducible manner. In one embodiment the endothelial cells of the invention can express the TM protein constitutively, i.e. continuously; thus the TM coding sequence is operably linked to a promoter sequence expressing the protein constitutively in said cell. Alternatively, the endothelial cells can express thrombomodulin on a regulable, e.g. inducible basis, i.e. the TM coding sequence is operably linked to an inducible promoter, such that the protein can be expressed immediately before or following endothelial cell activation, or on demand in response to a predetermined external stimulus. In a further embodiment, the TM cDNA can be operably linked to an endothelial cell specific promoter. All the above promoters are preferably other than the promoter which is native to the TM protein encoding DNA sequence.

Thus the invention in one aspect addresses the dysfunctional or activation response of vascular endothelial cells or tissue to an inflammatory or immune stimulus in a patient following such modification. It permits inhibition of thrombosis in a patient in need of such therapy following genetic modification of donor endothelial cells or tissue in vivo or in vitro by inserting therein DNA encoding thrombomodulin protein under the control of an appropriate, e.g. regulable or constitutive, promoter, grafting of these donor cells or tissue into a recipient, and thereby allowing expression of functional thrombomodulin from these donor cells or tissue.

The donor species may be any suitable species which is the same or different from the recipient species and which is able to provide the appropriate endothelial cells, tissue or organs for transplantation or grafting. In a preferred embodiment, human TM protein is expressed from cells of a different mammalian species, which cells have been placed or grafted into a human recipient. For human recipients, it is envisaged that pig donors will be suitable, but any other mammalian species, e.g. bovine, may be suitable. For example, porcine endothelial cells, or the progenitor cells thereof, can be recruited from porcine subjects, genetically modified, and implanted into either the autologous donor or into another mammalian, e.g. human subject.

The donor cells or tissue may be transgenic in the sense that they contain and express DNA encoding the thrombomodulin protein of a graft recipient species or of a different species in whom they are implanted. Such transgenic cells or tissue may continue to express thrombomodulin indefinitely for the life of the cell, tissue or organ.

Modification of endothelial cells according to the invention can be by any of various means known to the skilled worker. In vivo direct injection of cells or tissue with DNA can be carried out, for example. Methods of producing transgenic animals are becoming more widespread; appropriate methods of inserting foreign cells or DNA into animal tissue include microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection-k, transduction, retroviral infection, etc.

Retroviral vectors, and in particular, replication-defective retroviral vectors lacking one or more of the gag, pol, and env sequences required for retroviral replication, are well-known to the art and may be used to transform endothelial cells. PA317 or other producer cell lines producing helper-free viral vectors are welldescribed in the literature, e.g. in Miller and Buttimore, Mol. Cell. Biol. (1986), 2895-2902; USP 5'219'740; USP 4'861'719; USP 5'124'263; and USP 4'650'764.

A representative retroviral construct suitable for purposes of the invention comprises at least one viral long terminal repeat and promoter sequence upstream of the nucleotide sequence of the therapeutic substance, and at least one viral long terminal repeat and polyadenylation signal downstream of the therapeutic sequence. It can be, for example, the Moloney Murine Leukemia Virus (Mo-Mu-LV)-derived N2 pro-viral vector in which the gene of interest is flanked by a 5'- and a 3'- long terminal repeats (LTR's) and a packaging

signal sequence downstream of the 5'-LTR. N2 also contains approximately 400 base pairs of the gag coding region of Mo-Mu-MLV. Additionally, a large portion of the Mo-Mu-MLV coding sequence has been deleted in N2 and replaced with the bacterial neomycin-resistance gene (neo) [Eglitis et al., Science 230 (1985) 1395-1398].

Porcine endothelial cells, such as porcine aortic endothelial cells, have been difficult to manipulate by typical genetic engineering techniques. Various vector constructs for constitutive expression of thrombomodulin in mammalian cells are given below in Table 1. It has surprisingly been found that a particular retroviral construct provides superior viral titre as well as protein expression of the transduced thrombomodulin. The exemplifed **pNTK-2** vector has been found superior to e.g. the other retroviral contructs indicated in Table 1 below for delivering high levels of viral titre and thrombomodulin expression:

Table 1:

Vector	neo resistance gene driven by:	cDNA driven by
LXSN	SV 40 promoter/enhancer	5' LTR
pBC140	5LTR	CMV promoter/enhancer
pCMVLTRtkneo5	Thymidine kinase promoter	5' LTR
pNTK-2	5' LTR	Thymidine kinase promoter

CMV = cytomegalovirus
LXSN = Long terminal repeat - X (insert) - SV40 promoter - Neomycin phosphotransferase gene
SV = simian virus

As a result of an apparent synergy between the promoter-containing 5'-long terminal repeat sequence of the present pNTK-2 vector and its Herpes simplex tk promoter sequence, attractive thrombomodulin expression levels are obtainable from transformed endothelial cells.

Vectors derived from adenoviruses, i.e. viruses causing upper respiratory tract disease and also present in latent infections in primates, are also generally known and may be used. The ability of adenoviruses to attach to cells at low ambient temperatures is an advantage in

the transplant setting which can facilitate gene transfer during c ld preservation. Adenoviral vector constructs can be prepared in various ways to effect expression of thrombomodulin protein. In such vectors, the TM gene may be under the control f one or more different promoters. One such promoter is the known CMV promoter/enhancer.

An adenoviral vector for the delivery of TM into endothelial cells may e.g. be constructed as follows:

(a) A TM transfer vector can comprise the TM gene (e.g. construct TM.CW1) downstream and under control of the CMV promoter/enhancer with polyA sequences downstream of the gene. Flanking this construct are adenoviral sequences necessary to promote recombination with a suitable vector. For example, such sequences may comprise about 400 bp of the adenoviral sequence upstream and about 3 kb of the adenoviral sequence downstream, which is numbered approximately 3000-6000 relative to the upstream 400 bp sequence.

(b) An adenoviral vector construct comprising the adenoviral genome with an insert in the E1 coding region, whereby the insert prevents the plasmid from giving rise to background.

(c) 293 cells are a continuous line deriving from human fetal kidney, containing and expressing proteins of the adenoviral E1 region (i.e. in trans) necessary for replication and related functions in an adenovoral vector which lacks this region. For example, 293 cells can contain and express the adenoviral E1a and E1b proteins needed for replication-related functions.

The recombination of vector (a) and vector (b) by co-transfection into 293 cells results in formation of adenoviral particles containing the DNA encoding TM, these particles being preferably lacking in one or more sequences of the adenoviral region, and thus being preferably replication defective. Co-transfection is done while the cells are overlayed with medium containing 1 % agarose so that the pure recombinant virus or viral vectors are obtained directly as visible clearings/plaques in the monolayer upon lysing of virus producing cells. The recombinant virus may be characterized and expanded physically by, for example, PCR and Southern blot and by expression using e.g. huTM monoclonal antibody prepared by known procedures. The resulting replication-defective recombinant virus as obtained, for example, by freeze-thaw lysis of infected 293 cells, is particularly effective for constitutive production of TM protein, in particular in endothelial cells.

An adenoviral vector contruct for use in the invention comprises an expression cassette comprising the thrombomodulin DNA in perative association with the cmv promoter, and a poly A sequence downstream therefrom. The expression cassette is introduced into the adenovirus genome by recombination of two plasmids in human embryonic kidney cells (293 cell line) which prove the presence of E1 genes in trans and thus permit replication of an adenovirus vector which lacks the E1 region.

An alternative approach to targeted gene delivery is through the formation of a DNA-protein complex. This type of gene transfer substrate is constructed by conjugating the thrombomodulin gene to a polypeptide ligand for an acceptor on an endothelial cell.

Cells or cell populations can be treated in accordance with the present invention in vivo or in vitro. For example, for purposes of in vivo treatments, thrombomodulin vectors of the present invention can be inserted by direct infection of cells, tissues or organs in situ. Thus the vessels of an organ such as a kidney can be temporarily clamped off in vivo from the blood circulation and the blood vessels perfused with a solution comprising a transmissible vector construct containing the subject thrombomodulin gene, for a time sufficient for the gene to be inserted into cells of the organ; and, on removal of clamps, blood flow can then be restored to the organ. In another embodiment, cellular modification can be carried out ex vivo in cultured cells. Cell populations can be removed from the donor or patient, genetically modified by insertion of vector DNA, and then implanted into the patient or another recipient. Additionally, an organ can be removed from a donor, subjected ex vivo to the perfusion step described above, and the organ can be re-grafted into the donor or implanted in a different recipient who is of the same or a different species.

Genetically modified endothelial cells may be introduced e.g. in reseeded denuded vessels or prostheses at the site of a transplant. Tissue or organs comprised thereof may also be removed from a donor and grafted into a recipient by well-known surgical procedures. Prior to implantation, the treated endothelial cells or tissue may be screened for genetically modified cells containing and expressing the construct. For this purpose, the vector construct can also be provided with a second nucleotide sequence encoding an expression product that confers resistance to a selectable marker substance. Suitable

selectable markers for screening include the neo gene, conferring resistance to neomycin, or the neomycin analog G418.

Although any mammalian cells can be targeted for insertion of the thrombomodulin gene, endothelial cells are the preferred cells for manipulation. The recipient species will be primarily but not exclusively human. Other mammals, such as primates, may be suitable recipients.

An inflammatory stimulus which leads to activation of endothelial cells by serum factors such as TNF occurs in a wide variety of pathological conditions, including injuries, burns, septic shock, autoimmune disease, allogeneic or xenotransplantation transplantation, surgery, etc. Further diseases include those where there is an increase in propensity for thrombus formation (e.g. atherosclerotic and thrombotic conditions such as ischemic heart disease, atherosclerosis, multiple sclerosis, intracranial tumors, thromboembolism and hyperlipemia, thrombophlebitis, phlebothrombosis, cerebral thrombosis, coronary thrombosis and retinal thrombosis), as well as those following parturition or surgical operations such as coronary artery bypass surgery, angioplasty and prosthetic heart valve implantation.

Insofar as the preparation of the reagents, starting materials or cell lines mentioned is not specifically described herein, they are known and available or they, or equivalents thereof, may be prepared from known available products according to known procedures.

The following Example is by way of illustration only and is not intended to be limitative in any respect of the invention herein described and claimed. All temperatures are in degrees Centigrade.

Example

Materials

Human thrombomodulin cDNA is obtained from the American Type Culture Collection (Rockville, MD, USA) cloned in the EcoRI site of plasmid pUC19 to form pUC19TM15 (ATCC Accession No. 61348).

Plasmid **pNTK-2** is derived from **pXT1** [Boulter et al., Nucleic Acids Research 15 (17) (1987) 7194, and further described in **Fig. 1**.

The **PA317** cell line (ATCC CRL 9078) contains an amphotropic, replication-defective retroviral construct lacking in the viral packaging signal but encoding the gag, pol, and env protein packaging genes.

Pig aortic endothelial cells (**PAEC**) are purified from aorta obtained e.g. from the Deaconess Hospital Animal Laboratories (Boston, MA, USA). Passage 4 human umbilical vein endothelial cells (**HUVEC**) originate from Children's Hospital (Boston, MA, USA).

The PA317 cells and PAEC are maintained on DMEM media (Gibco) with 10% heat-inactivated fetal calf serum, penicillin and streptomycin.

Methods

a. Construction of pNTK-2.TM.CW1

Digestion of plasmid **pUC19.TM15** with BstXI and HindIII produces a truncated cDNA sequence from which the 3'-untranslated region, including polyadenylation sites of the TK gene and a small segment of the adjacent pUC polylinker, are deleted. The excised segment is replaced by a synthetic linker of 29 nucleotides plus overhangs encoding intermediate SalI, EcoR1 and BamHI sites, and is schematically depicted in **FIG. 2b** as **pUC.TM.CW1**.

The truncated sequence TM.CW1 is cut out of pUC.TM.CW1 at the Sal-1 site and cloned into **pNTK-2** as a SalI fragment fused to the XhoI site in pNTK-2 to give **pNTK-2.TM.CW1**, schematically depicted in **FIG. 3**.

b. Transduction of producer cells

Plasmid pNTK-2.TM.CW1 is linearized in the plasmid backbone with ScaI, and the

linearized plasmid pNTK-2.TM.CW1 is transduced by electroporation into PA 317 cell culture.

c. Control producers

A plasmid is prepared by the methods described under a. above, except that it is lacking the thrombomodulin gene. This plasmid is also transduced by electroporation into PA317 cells to produce "empty" provirus, i.e. viral titres containing the neo gene but not the thrombomodulin gene.

d. Selection and analysis of producer clones

Neomycin selection of the transfected PA317 cells is carried out for at least 14 days in at least 600 µg/ml G418 (Gibco Grand Island NY Cat. No. 11811-031). The resulting NTK-2.TM.CW1 producer pool is sub-cloned by limiting dilution to yield 29 sub-clones. Supernatant from the sub-clones is harvested in the late log, early stationary phase and spun at 2000 g for 10 minutes to remove producer cells and debris, then purified in a single cycle of rapid freeze-thawing in liquid nitrogen.

e. FACscan analysis

FACscan analysis of the NTK-2.TM.CW1 producer pool is carried out using anti-thrombomodulin monoclonal antibody 11B1 and FITC conjugated secondary reagents according to the procedure described by Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Publication, Ch. 10 (1988). In FIG. 4a, the unfilled area shows producer cells incubated with the secondary reagent alone. The filled area (secondary reagent *and* 11 B1) corresponds to the level of thrombomodulin per cell, increasing left to right. The NTK-2.TM.CW1 producer pool produces a medium titre virus (1-2 x 10^4 infectious units/ml). Approximately 80% of the NTK-2.TM.CW1 clones are found to be TM expressing. 10-15% of the subclones produce virus which confers G418 resistance but not NTK-2.TM.CW1 expression (FIG. 4b).

f. Infection of 3T3 cells

3T3 fibroblasts are infected with purified viral supernatant and selected with G418 14 days thereafter. Of the 29 sub-clones, 22 produce viral titres on the 3T3 fibroblasts. By comparison, other retroviral constructs containing NTK-2.TM.CW1 under the control of

stronger constitutive promoters, such as hereinabove described in Table 1, produce low viral titres. Selection of a single clone, A5, results in an approximate doubling of the transduction frequency as judged by anti-TM immunohistochemistry, from 30-40% to 60-80%, respectively.

g. Infection of PAEC

PAEC in the log phase of growth are seeded at 0.5 - 2 x 10^9 cells per 30 mm-diameter petri dish and incubated with the purified A5 supernatant in the presence of 8 µg/ml polybrene surfactant over 24 hours.

h. PAEC stimulation with bacterial endotoxin and human TNF

Confirmation that the transduced PAEC retains susceptibility to activation is obtained by carrying out an ELISA with anti-human TM monoclonal antibody 1A4 on PAEC clones treated with 100 ng/ml of bacterial cell wall lipopolysaccharide (LPS) or TNF, known cell activators. The cells respond to stimulation by up-regulating E-selectin, which is known to be up-regulated during cellular activation. Stimulation results in some reduction of thrombomodulin activity, as also measured by ELISA with 1A4, which appears largely attributable to posttranslational mechanisms (i.e. the oxidation tendency of $methionine_{388}$ of the protein) rather than reduced expression. TM expression and APC production in response to stimulus of various cell lines with TNF is shown in **FIG. 9** (error bars ± 1 standard deviation). The activity of the endogenous TM protein expressed by HUVEC is essentially fully down-regulated by TNF-α, whereas human TM expressed by transduced PAEC is resistant to cytokine-induced down-regulation.

i. Immunoperoxidase study

The transduction efficiency of exponentially growing PAEC is determined 24 hours after infection by indirect immunoperoxidase staining with antibody 1A4. The transduced PAEC are washed with phosphate buffered saline (PBS), then fixed for 10 minutes with 0.05 % glutaraldehyde at room temperature. After blocking in 5 % goat serum in PBS, the cells are incubated for 1 hour in 2 % goat serum in PBS at room temperature with anti-human thrombomodulin monoclonal 1A4 diluted 1:350; biotinylated goat anti-mouse antibody (Pierce 31802, diluted 1:2000) and streptavidin peroxidase (Pierce, diluted 1 :2000). The presence of antigen was detected using aminoethyl-carbazole. Good

transduction frequencies are obtained with a single round of infection using supernatant harv sted from the A5 clone. In untransduced PAEC, thrombomodulin message is negligable and no protein is detected.

j. Northern analysis

Total RNA is then isolated from the transduced, positive-selected PAEC by the guanidinium isothiocyanate / acid phenol method. **FIG. 5** shows the Northern blot of RNA (16 h exposure) from PAEC culture cells probed with the EcoRI fragment of pUC.TM.CW1; untransduced PAEC; EAhy926; NIH 3T3; and NTK-2.TM.CW1 transduced, positive selected **NIH3T3**. The lanes contain 10 µg RNA. The interpolated size of thrombomodulin mRNA is 3.7 kilobases.

Two major RNA species predominate: (1) a 3-3.5 kb sequence, corresponding to the tk driven transcript; and (2) an approximately 5.5 kb sequence hybridizable with a TM probe and a probe for G418 resistance, evidencing transcriptional readthrough, starting with the LTR driven transcription and continuing from the neo gene through the tk and TM sequence, corresponding to a 5'-LTR to 3'LTR transcript.

k. Nuclease S1 analysis

Steady state levels of human TM-encoding mRNA are determined by S1 nuclease protection analysis. TM15 cloned in plasmid **pKS M13+** at the EcoRI site is digested with Pvull and Xmal to produce a 530 bp probe for S1 nuclease protection. The protected fragments seen in the transduced cells correspond to the 260 bp fragment that would be expected from the EcoRI site to the Xmal site in TM15. The wild type human thrombomodulin yields a protected fragment 23 bp shorter. S1 nuclease analysis (3 days exposure) of RNA from transduced cells demonstrates significant steady-state expression of human TM-encoding mRNA (**FIG. 6**).

l. Scatchard analysis of TM expression.

A comparison is made between the level of expression of TM in transduced PAEC as compared with the high-expressing **EAhy926** cell line, and with human umbilical cord vein endothelial cells (HUVEC), which expresses human TM constitutively.

The comparison is made by first estimating the absolute number of thrombomodulin molecules per cell on the EAhy926 cell line since all cells should express the molecule,

using Scatchard analysis with radiolabelled 1A4. The levels of expression of the PAEC and EAhy926 are of similar magnitude. In a comparative ELISA, the EAhy926 cells express an average of 2.5 x 10^5 molecules per cell, and the G418-selected NTK-2.TM.CW1 - transduced cells at least 1.5x 10^5 molecules per cell.

m. Biological activity

Functionality of expressed human TM is quantified by co-factor assay on confluent human TM-expressing PAEC, empty vector-transduced PAEC, HUVEC and EAhy926 (**FIG. 7**). The cells are grown to confluence in 24-well plates. The culture medium is removed and wells washed with PBS. The cell cultures are incubated with 170 µl of the following substrate co-factor solution:

0.8 µg human Protein C (purified from pooled plasma (American Red Cross)
0.1 international units of bovine thrombin in 1x cofactor buffer (comprising 50 mM tris-HCl, pH 8.0; 100 mM NaCl; 10 mM $CaCl_2$; 0.1% BSA),

for 60 minutes at 37° in a CO_2 incubator as described in Owen, W. and Esmon, C., J. Biol. Chem. 257 (1982) 859 and Freyssinet et al., Biochem. J. 238 (1986) 151. To quench the reaction, 3 units of leech hirudin (a thrombin binder) is added to each culture. Activated Protein C generated during the above incubation is assayed kinetically using the chromogenic substrate, **S-2366** by combining 159 µl of quenched reaction mixture and 50 µl of substrate and reading the OD at 405 nm in a microplate reader. Activity of the thrombomodulin is expressed as the maximum rate of cleavage of the chromogen in mOD units/min. The thrombomodulin expressed by the PAEC is found able to catalyze thrombin mediated action of human protein C, using both human and bovine thrombin. By comparison, no significant thrombomodulin activity is detected on the untransduced PAEC (**FIG. 8**), and less activity is seen with the EAhy926 cell line.

The activity of the thrombomodulin is comparable to that seen with HUVEC.

n. Clotting studies

A microwell in vitro clotting experiment can demonstrate that transduced PAEC causes an extension of clotting times when activated with human plasma followed by freeze-thaw disruption of the cells.

Sequence listing

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Sandoz Ltd.
(B) STREET: Lichtstrasse 35
(C) CITY: Basle
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): CH-4002
(G) TELEPHONE: 61-324 5269
(H) TELEFAX: 61-322 7532

(A) NAME: New England Deaconess Hospital Corporation
(B) STREET: 185 Pilgrim Road
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 02215

(A) NAME: BACH, Fritz H.
(B) STREET: 8, Blossom Lane
(C) CITY: Manchester-By-The-Sea, Boston
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 01966

(A) NAME: WRIGHTON, Christopher
(B) STREET: 17, Stearns Road, Unit 5
(C) CITY: Brookline
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 02146

(ii) TITLE OF INVENTION: GENE THERAPY FOR TRANSPLANTATION AND INFLAMMATORY OR THROMBOTIC CONDITIONS

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/EP 95/.....

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/296'945
(B) FILING DATE: 26-AUG-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1946 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 4'912'207 P
(I) FILING DATE: 06-MAY-1987
(J) PUBLICATION DATE: 27-MAR-1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCTAGA GTCGACTGCT ACCGGCAGCG CGCAGCGGCA AGAAGTGTCT GGGCTGGGAC 60

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GCAGTGCCCG CGCTTTCCCC GGCGCCTGCA CGCGGCGCGC CTGGGTAACA TGCTTGGGGT 180

CCTGGTCCTT GGCGCGCTGG CCCTGGCCGG CCTGGGGTTC CCCGCACCCG CAGAGCCGCA 240

GCCGGGTGGC AGCCAGTGCG TCGAGCACGA CTGCTTCGCG CTCTACCCGG GCCCCGCGAC 300

CTTCCTCAAT GCCAGTCAGA TCTGCGACGG ACTGCGGGGC CACCTAATGA CAGTGCGCTC 360

CTCGGTGGCT GCCGATGTCA TTTCCTTGCT ACTGAACGGC GACGGCGGCG TTGGCCGCCG 420

GCGCCTCTGG ATCGGCCTGC AGCTGCCACC CGGCTGCGGC GACCCCAAGC GCCTCGGGCC 480

CCTGCGCGGC TTCCAGTGGG TTACGGGAGA CAACAACACC AGCTATAGCA GGTGGGCACG 540

GCTCGACCTC AATGGGGCTC CCCTCTGCGG CCCGTTGTGC GTCGCTGTCT CCGCTGCTGA 600

GGCCACTGTG CCCAGCGAGC CGATCTGGGA GGAGCAGCAG TGCGAAGTGA AGGCCGATGG 660

CTTCCTCTGC GAGTTCCACT TCCCAGCCAC CTGCAGGCCA CTGGCTGTGG AGCCCGGCGC 720

CGCGGCTGCC GCCGTCTCGA TCACCTACGG CACCCCGTTC GCGGCCCGCG GAGCGGACTT 780

CCAGGCGCTG CCGGTGGGCA GCTCCGCCGC GGTGGCTCCC CTCGGCTTAC AGCTAATGTG 840

CACCGCGCCG CCCGGAGCGG TCCAGGGGCA CTGGGCCAGG GAGGCGCCGG GCGCTTGGGA 900

CTGCAGCGTG GAGAACGGCG GCTGCGAGCA CGCGTGCAAT GCGATCCCTG GGGCTCCCCG 960

CTGCCAGTGC CCAGCCGGCG CCGCCCTGCA GGCAGACGGG CGCTCCTGCA CCGCATCCGC 1020

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CTCCTACTCG TGCATGTGCG AGACCGGCTA CCGGCTGGCG GCCGACCAAC ACCGGTGCGA 1140

GGACGTGGAT GACTGCATAC TGGAGCCCAG TCCGTGTCCG CAGCGCTGTG TCAACACACA 1200

GGGTGGCTTC GAGTGCCACT GCTACCCTAA CTACGACCTG GTGGACGGCG AGTGTGTGGA 1260

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TAGCTACCTC TGCGTCTGCG CCGAGGGCTT CGCGCCCATT CCCCACGAGC CGCACAGGTG 1380

CCAGATGTTT TGCAACCAGA CTGCCTGTCC AGCCGACTGC GACCCCAACA CCCAGGCTAG 1440

CTGTGAGTGC CCTGAAGGCT ACATCCTGGA CGACGGTTTC ATCTGCACGG ACATCGACGA 1500

GTGCGAAAAC GGCGGCTTCT GCTCCGGGGT GTGCCACAAC CTCCCCGGTA CCTTCGAGTG 1560

CATCTGCGGG CCCGACTCGG CCCTTGCCCG CCACATTGGC ACCGACTGTG ACTCCGGCAA 1620

GGTGGACGGT GGCGACAGCG GCTCTGGCGA GCCCCCGCCC AGCCCGACGC CCGGCTCCAC 1680

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CGCCGCCAGG GCCAAGATGG AGTACAAGTG CGCGGCCCCT TCCAAGGAGG TAGTGCTGCA 1860

GCACGTGCGG ACCGAGCGGA CGCCGCAGAG ACTCTGAGCG GCCTCCGTCC AGGAGCGTCG 1920

TCGACGAATT CGGATCCGAC AAGCTT                                       1946
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Claims

1. A method of genetically modifying mammalian cells to render them less susceptible to an inflammatory or immunological stimulus, comprising inserting therein, or in the progenitors thereof, DNA encoding functional thrombomodulin protein in operative association with a suitable promoter, whereby thrombomodulin is expressed under endothelial cellular activating conditions.

2. A method of inhibiting thrombosis in a mammalian subject susceptible to an inflammatory or immune stimulus which comprises inserting into cells, tissue or an organ of the subject, DNA encoding thrombomodulin in operative association with a promoter, whereby functional thrombomodulin is expressed from these modified cells in the presence of cellular activating factors.

3. The method of claim 1 or 2 in which the DNA encoding mammalian thrombomodulin is inserted into endothelial cells or tissue.

4. The method of claim 3 in which the DNA encodes human thrombomodulin.

5. The method of claim 4 in which the DNA encoding thrombomodulin is in operative association with a constitutive promoter.

6. The method of claim 4 in which the DNA encoding thrombomodulin is in operative association with a regulable and/or inducible promoter.

7. The method of claim 1 or 2 in which the DNA encoding thrombomodulin is in operative association with the thymidine kinase promoter.

8. The method of claim 1 or 2 in which the DNA encoding thrombomodulin is in operative association with both the thymidine kinase promoter and a retroviral 5'-long terminal repeat DNA sequence, this repeat sequence being located upstream from the thymidine kinase promoter.

9. The method of claim 1 or 2 in which a retroviral 3'-long terminal repeat DNA sequence is downstream from the DNA encoding thrombomodulin.

10. The method of claim 1 or 2 in which the DNA encoding thrombomodulin is in operative association with the cmv promoter.

11. A method of transplanting donor allogeneic or xenogeneic endothelial cells, or tissue or organs containing endothelial cells to a mammalian recipient in whom such cells, tissue or organ are subject to inflammatory or immune activation, which comprises:

(a) genetically modifying these donor cells or progenitor cells thereof or tissue or organ by inserting therein DNA encoding thrombomodulin under the control of a promoter;

(b) implanting the resultant modified donor cells, tissue or organ into the recipient; and

(c) allowing expression from the resultant modified cells, tissue or organ of functionally active thrombomodulin.

12. The method of claim 11 in which DNA encoding human thrombomodulin is inserted into the donor cells or tissue and the recipient is a human.

13. The method of claim 12 in which the donor cells or tissue are of a non-human mammal.

14. The method of claim 13 in which the DNA encoding thrombomodulin is in operative association with a constitutive promoter.

15. The method of claim 13 in which the DNA encoding thrombomodulin is in operative association with a regulable and/or inducible promoter.

16. The method of claim 13 in which the DNA encoding thrombomodulin is in operative association with the thymidine kinase promoter.

17. The method of claim 16 in which the DNA encoding thrombomodulin is in operative association with both the thymidine kinase promoter and a retroviral 5'-long terminal repeat DNA sequence, the repeat sequence being located upstream of the thymidine kinase promoter.

18. The method of claim 17 in which a retroviral 3'-long terminal repeat DNA sequence is downstream from the DNA encoding thrombomodulin.

19. The method of claim 13 in which the DNA encoding thrombomodulin is in operative association with the cmv promoter.

20. A retroviral construct which comprises:

(a) a 5'-long terminal repeat (LTR) of a retrovirus;

(b) a retroviral packaging signal downstream from the LTR;

(c) DNA encoding thrombomodulin in operative association with the Herpes simplex thymidine kinase promoter downstream from the LTR;

(d) a 3'-long terminal repeat.

21. Graftable mammalian endothelial cells, tissue or organs comprising DNA encoding thrombomodulin under the control of a constitutive, or a regulable and/or inducible promoter.

22. The endothelial cells, tissue or organs of claim 21 of a donor mammalian species, the cells, tissue or organs being modified to express thrombomodulin of a graft recipient species which is a different mammalian species as the donor.

23. The cells, tissue or organs of claim 22 wherein the donor mammalian species is porcine and the graft recipient is human.

24. A non-human transgenic mammal comprising DNA encoding thromb modulin of a different species.

25. The transgenic mammal of claim 24 which is a pig or a mouse and in which the DNA encodes human thrombomodulin.

fig. 1

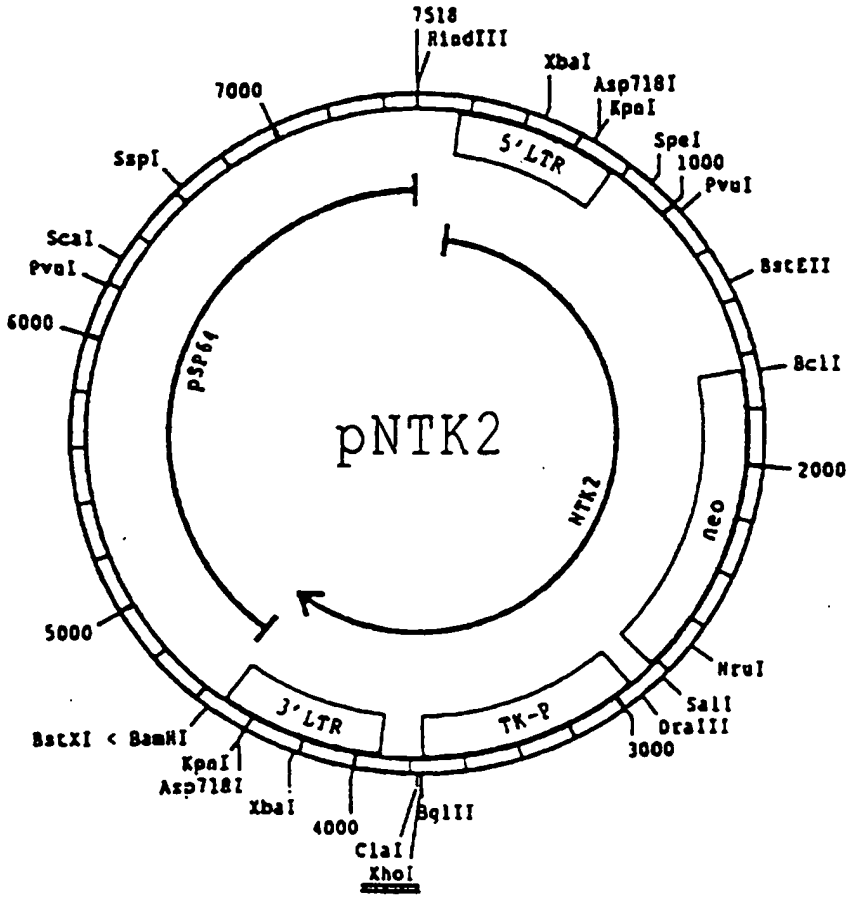

7518
HindIII
XbaI
Asp718I
KpnI
SpeI
1000
PvuI
BstEII
BclI
2000
NruI
SalI
OraIII
3000
BglII
ClaI
XhoI
4000
XbaI
Asp718I
KpnI
BstXI < BamHI
5000
ScaI
PvuI
6000
SspI
7000
5'LTR
TK-P
3'LTR
neo
NTK2
PSP64
pNTK2

fig. 2a

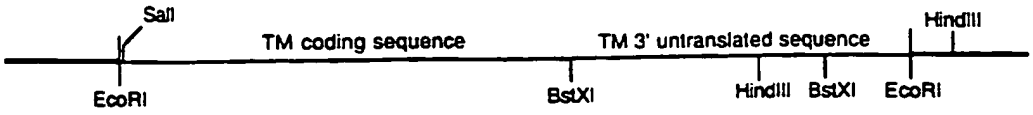

SalI
TM coding sequence
TM 3' untranslated sequence
HindIII
EcoRI
BstXI
HindIII
BstXI
EcoRI

FIG. 2b


SalI
TM coding sequence
SalI EcoRI BamHI
EcoRI
BstXI
HindIII

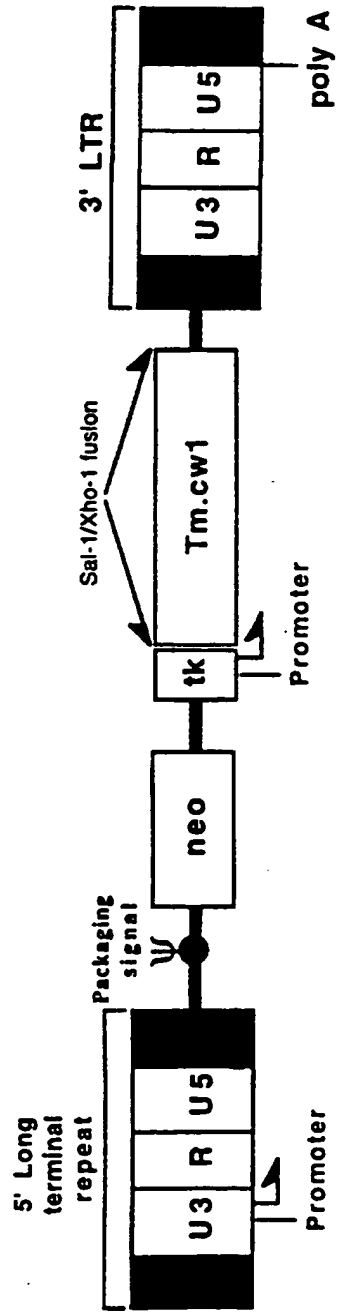

3' LTR
U3
R
U5
poly A
Sal-1/Xho-1 fusion
Tm.cw1
tk
Promoter
neo
Packaging signal
Ψ
5' Long terminal repeat
U3
R
U5
Promoter

FIG. 3

FIG. 4a

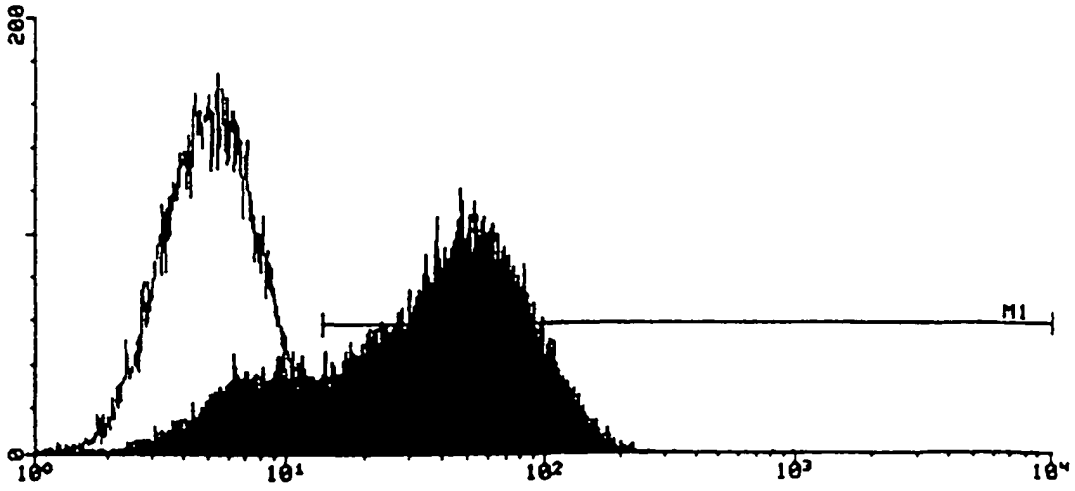

200
0
M1
10^0
10^1
10^2
10^3
10^4

FIG. 4b

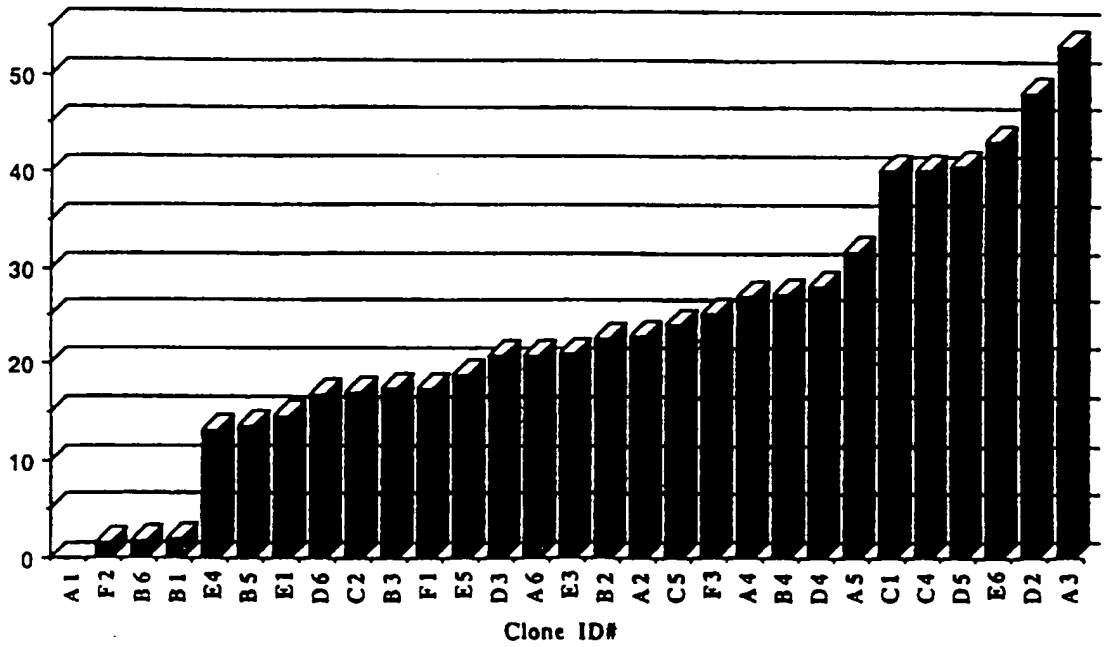

50
40
30
20
10
0
A1 F2 B6 B1 E4 B5 E1 D6 C2 B3 F1 E5 D3 A6 E3 B2 A2 C5 F3 A4 B4 D4 A5 C1 C4 D5 E6 D2 A3
Clone ID#

FIG. 5

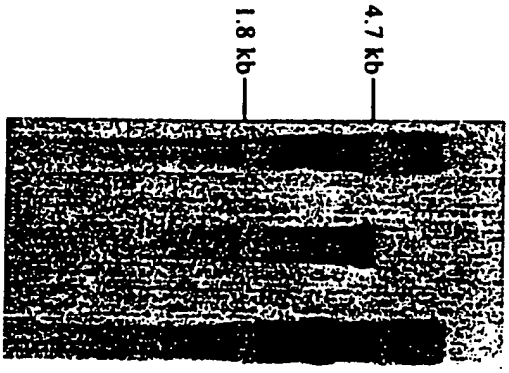

1.8 kb
4.7 kb
NTK.TM.CW1 transduced+selected PAEC
Untransduced PAEC
EAhy 926
NIH 3T3
NTK.TM.CW1 transduced+selected NIH 3T3

FIG. 6

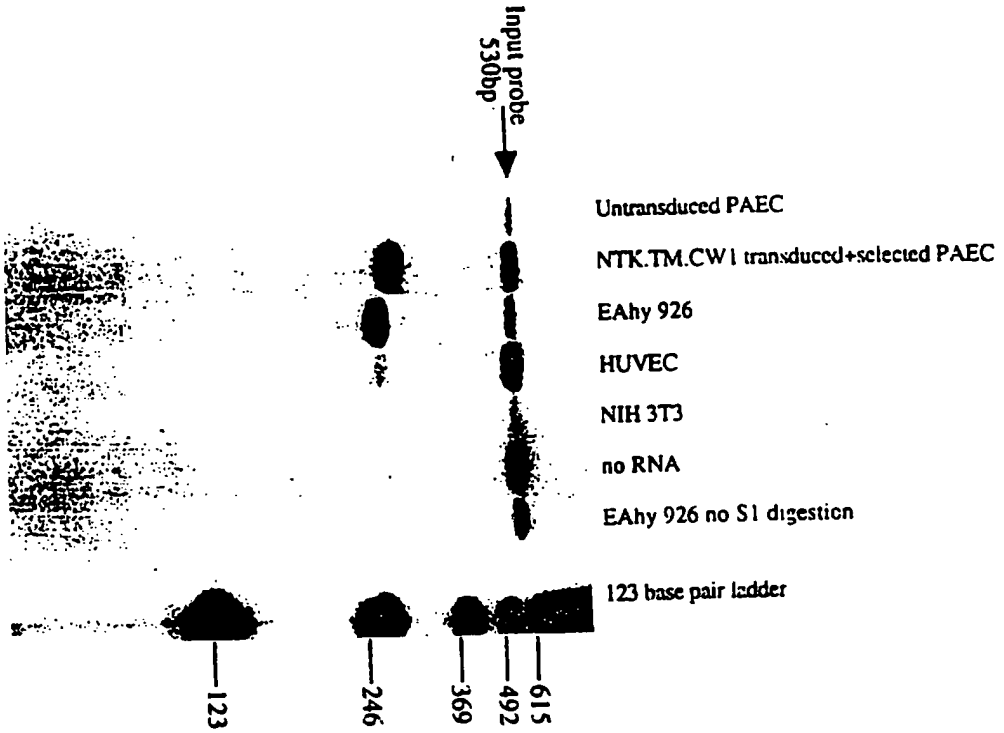

Input probe
530bp
Untransduced PAEC
NTK.TM.CW1 transduced+selected PAEC
EAhy 926
HUVEC
NIH 3T3
no RNA
EAhy 926 no S1 digestion
123 base pair ladder
123
246
369
492
615

fig. 7

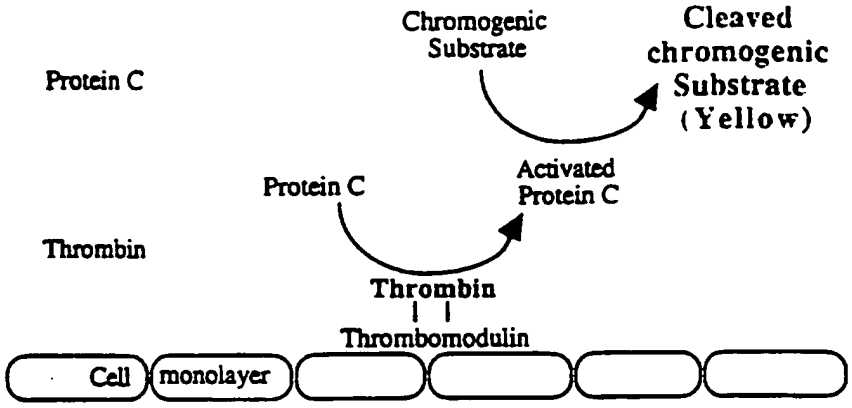

Chromogenic Substrate
Cleaved chromogenic Substrate (Yellow)
Protein C
Protein C
Activated Protein C
Thrombin
Thrombin
Thrombomodulin
Cell
monolayer

fig. 8

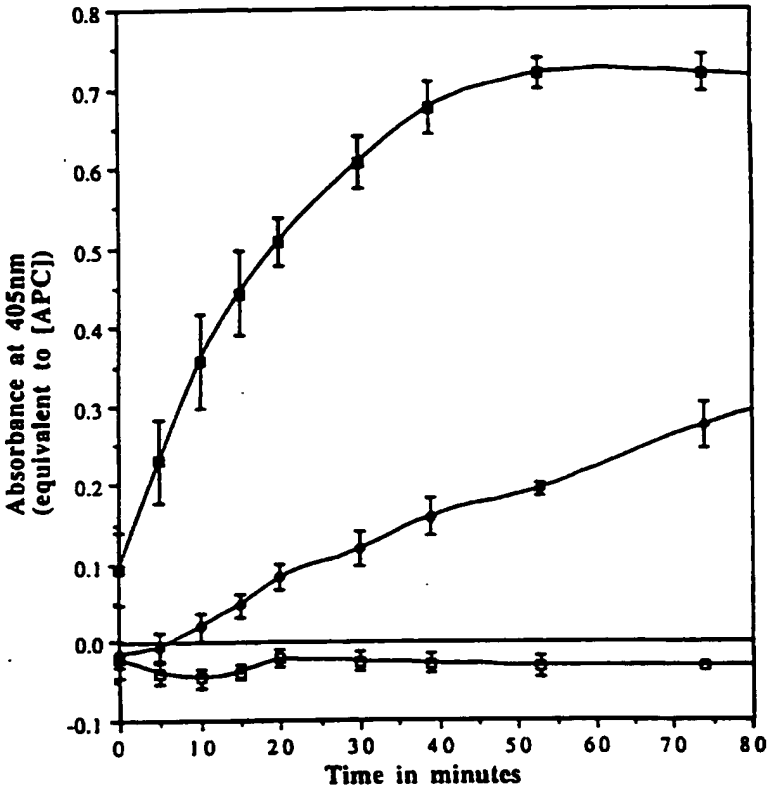

Absorbance at 405nm
(equivalent to [APC])
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
10
20
30
40
50
60
70
80
Time in minutes

FIG. 9

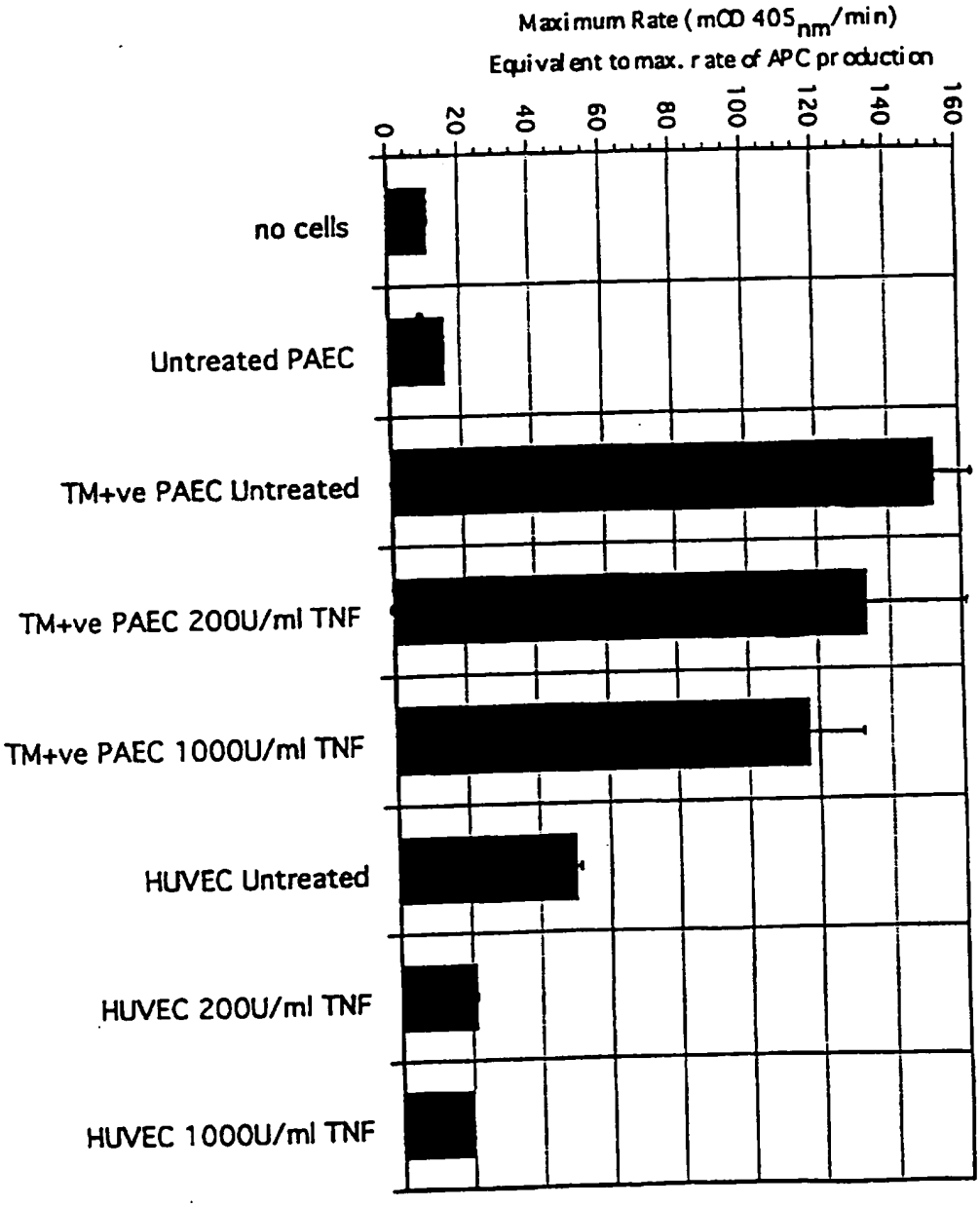

Maximum Rate (mOD 405nm/min)
Equivalent to max. rate of APC production
0
20
40
60
80
100
120
140
160
no cells
Untreated PAEC
TM+ve PAEC Untreated
TM+ve PAEC 200U/ml TNF
TM+ve PAEC 1000U/ml TNF
HUVEC Untreated
HUVEC 200U/ml TNF
HUVEC 1000U/ml TNF

FIG. 10

```
   1 GAATTCTAGA GTCGACTGCT ACCGGCAGCG CGCAGCGGCA AGAAGTGTCT GGGCTGGGAC
  61 GGACAGGAGA GGCTGTCGCC ATCGGCGTCC TGTGCCCCTC TGCTCCGGCA CGGCCCTGTC
 121 GCAGTGCCCG CGCTTTCCCC GGCGCCTGCA CGCGGCGCGC CTGGGTAACA TGCTTGGGGT
 181 CCTGGTCCTT GGCGCGCTGG CCCTGGCCGG CCTGGGGTTC CCCGCACCCG CAGAGCCGCA
 241 GCCGGGTGGC AGCCAGTGCG TCGAGCACGA CTGCTTCGCG CTCTACCCGG GCCCCGCGAC
 301 CTTCCTCAAT GCCAGTCAGA TCTGCGACGG ACTGCGGGGC CACCTAATGA CAGTGCGCTC
 361 CTCGGTGGCT GCCGATGTCA TTTCCTTGCT ACTGAACGGC GACGGCGGCG TTGGCCGCCG
 421 GCGCCTCTGG ATCGGCCTGC AGCTGCCACC CGGCTGCGGC GACCCCAAGC GCCTCGGGCC
 481 CCTGCGCGGC TTCCAGTGGG TTACGGGAGA CAACAACACC AGCTATAGCA GGTGGGCACG
 541 GCTCGACCTC AATGGGGCTC CCCTCTGCGG CCCGTTGTGC GTCGCTGTCT CCGCTGCTGA
 601 GGCCACTGTG CCCAGCGAGC CGATCTGGGA GGAGCAGCAG TGCGAAGTGA AGGCCGATGG
 661 CTTCCTCTGC GAGTTCCACT TCCCAGCCAC CTGCAGGCCA CTGGCTGTGG AGCCCGGCGC
 721 CGCGGCTGCC GCCGTCTCGA TCACCTACGG CACCCCGTTC GCGGCCCGCG GAGCGGACTT
 781 CCAGGCGCTG CCGGTGGGCA GCTCCGCCGC GGTGGCTCCC CTCGGCTTAC AGCTAATGTG
 841 CACCGCGCCG CCCGGAGCGG TCCAGGGGCA CTGGGCCAGG GAGGCGCCGG GCGCTTGGGA
 901 CTGCAGCGTG GAGAACGGCG GCTGCGAGCA CGCGTGCAAT GCGATCCCTG GGGCTCCCCG
 961 CTGCCAGTGC CCAGCCGGCG CCGCCCTGCA GGCAGACGGG CGCTCCTGCA CCGCATCCGC
1021 GACGCAGTCC TGCAACGACC TCTGCGAGCA CTTCTGCGTT CCCAACCCCG ACCAGCCGGG
1081 CTCCTACTCG TGCATGTGCG AGACCGGCTA CCGGCTGGCG GCCGACCAAC ACCGGTGCGA
```

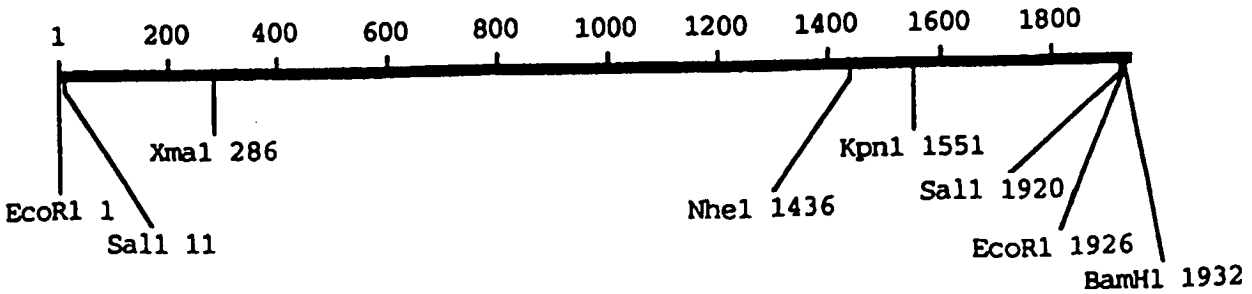
-10/10 (beginning) -

FIG. 10 (end)

```
1141 GGACGTGGAT GACTGCATAC TGGAGCCCAG TCCGTGTCCG CAGCGCTGTG TCAACACACA
1201 GGGTGGCTTC GAGTGCCACT GCTACCCTAA CTACGACCTG GTGGACGGCG AGTGTGTGGA
1261 GCCCGTGGAC CCGTGCTTCA GAGCCAACTG CGAGTACCAG TGCCAGCCCC TGAACCAAAC
1321 TAGCTACCTC TGCGTCTGCG CCGAGGGCTT CGCGCCCATT CCCCACGAGC CGCACAGGTG
1381 CCAGATGTTT TGCAACCAGA CTGCCTGTCC AGCCGACTGC GACCCCAACA CCCAGGCTAG
1441 CTGTGAGTGC CCTGAAGGCT ACATCCTGGA CGACGGTTTC ATCTGCACGG ACATCGACGA
1501 GTGCGAAAAC GGCGGCTTCT GCTCCGGGGT GTGCCACAAC CTCCCCGGTA CCTTCGAGTG
1561 CATCTGCGGG CCCGACTCGG CCCTTGCCCG CCACATTGGC ACCGACTGTG ACTCCGGCAA
1621 GGTGGACGGT GGCGACAGCG GCTCTGGCGA GCCCCCGCCC AGCCCGACGC CCGGCTCCAC
1681 CTTGACTCCT CCGGCCGTGG GGCTCGTGCA TTCGGGCTTG CTCATAGGCA TCTCCATCGC
1741 GAGCCTGTGC CTGGTGGTGG CGCTTTTGGC GCTCCTCTGC CACCTGCGCA AGAAGCAGGG
1801 CGCCGCCAGG GCCAAGATGG AGTACAAGTG CGCGGCCCCT TCCAAGGAGG TAGTGCTGCA
1861 GCACGTGCGG ACCGAGCGGA CGCCGCAGAG ACTCTGAGCG GCCTCCGTCC AGGAGCGTCG
1921 TCGACGAATT CGGATCCGAC AAGCTT
```

- 10/10(end) -

Restriction Analysis - HUMTHM CW1



INTERNATIONAL SEARCH REPORT

Internat. Application No
PCT/EP 95/03379

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/86 A61K48/00 C12N5/10 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 06997 (US GOVERNMENT ;UNIV MIAMI (US)) 28 June 1990 see the whole document ---	1-20, 22-25
Y	WO,A,92 07573 (SOMATIX THERAPY CORP ;HUGHES HOWARD MED INST (US); WHITEHEAD BIOME) 14 May 1992 see the whole document ---	1-20, 22-25
Y	WO,A,93 18794 (GENSIA PHARMA) 30 September 1993 see whole document,noting especially page 13, line 5-page, line 3 ---	1-20, 22-25
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
14 December 1995	11.01.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Sitch, W

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	see page 1391, summary, and first paragraph	1-20, 22-25

X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 24, 17 June 1994 pages 16925-16932, DITTMAN ET AL 'CHARACTERIZATION OF THROMBOMODULIN EXPRESSION IN RESOPNSE TO RETINOIC ACID AND IDENTIFICATION OF A RETINOIC ACID RESPONSE ELEMENT IN THE HUMAN THROMBOMODULIN GENE'	21
Y	see summary, page 16925, and first paragraph	1-20, 22-25

X	JOURNAL OF VASCULAR SURGERY, vol. 14, no. 5, November 1991 pages 599-604, SOLIS ET AL 'INTRAVENOUS RECOMBINANT SOLUBLE HUMAN THROMBOMODULIN PREVENTS VENOUS THROMBOSIS IN A RAT MODEL'	21
Y	see summary, page 599	1-20, 22-25

Y	DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US ABSTRACT 92:300032, LABARRERE ET AL 'NATURAL ANTICOAGULANT PATHWAYS IN NORMAL AND TRANSPLANTED HUMAN HEARTS' & J HEART LUNG TRANSPLANT 11 (2 PART 1).1992.342-347 see abstract	1-20, 22-25

P,X	WO,A,94 27612 (BAYLOR COLLEGE MEDICINE) 8 December 1994 see page 8, line 16 - page 11, line 4 see page 26, line 2 - page 32, line 4	1-7, 10-16, 19,21-25

P,X	WO,A,95 00654 (BETH ISRAEL HOSPITAL) 5 January 1995 see page 5, paragraph 3 - page 11, paragraph 1 see page 12, paragraph 1 - page 34, paragraph 1	1-25

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INTERNATIONAL SEARCH REPORT

Interna[illegible]l Application No
PCT/EP 95/03379

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	TRANSPLANTATION PROCEEDINGS, vol. 27, no. 1, February 1995 pages 288-289, WRIGHTON ET AL 'HIGH-LEVEL EXPRESSION OF FUNCTIONAL HUMAN THROMBOMODULIN IN CULTURED PORCINE AORTIC ENDOTHELIAL CELLS' see the whole document ---	1-25
E	WO,A,95 27512 (BAYLOR COLLEGE MEDICINE) 19 October 1995 see page 7, line 18 - page 27, line 31 -----	1-6,9, 10,20,24

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INTERNATIONAL SEARCH REPORT

...rmation on patent family members

Interna ...l Application No
PCT/EP 95/03379

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		CA-A-	2134237	30-09-93
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WO-A-9527512	19-10-95	NONE		